SPECIFIC ALANINE-tRNA SPECIES ASSOCIATED WITH FIBROIN BIOSYNTHESIS IN THE POSTERIOR SILK-GLAND OF *BOMBYX MORI* L.

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1. Introduction

During the course of our study on the role and mechanism of specific tRNA changes (tRNA adaptation) during the silk-fibroin secretion phase of the posterior silk-gland development in *Bombyx mori* L., we undertook fractionation and purification of the preponderant tRNAs, $tRNA^{Ala}$, $tRNA^{Gly}$, $tRNA^{Met}$, $tRNA^{Ser}$ and $tRNA^{Tyr}$, by benzoylated DEAE-cellulose column chromatography, countercurrent distribution and polyacrylamide gel electrophoresis [2–11]. We observed major differences in compartmentalization of isoaccepting $tRNA^{Ala}$ species in the two portions of the silk-gland, the posterior and middle silk-glands. Silk-fibroin (29% alanine, 46% glycine and 12% serine) is synthesized exclusively in the posterior part, whereas sericin (6% alanine, 17% glycine and 37% serine) is produced in the middle part of silk-gland. The predominant $tRNA^{Ala}$ species, $tRNA^{Ala}_{2a}$ and $tRNA^{Ala}_{2b}$, in the ratio of 2 : 1 (14% and 7% of the total tRNA content, respectively) are present in the posterior silk-gland during the fibroin secretion phase of gland development, whereas only one, $tRNA^{Ala}_{2b}$, species is the main $tRNA^{Ala}$ component of the middle silk-gland and of the carcass-tissue; the latter contains proteins of more uniform amino acid composition. Although both $tRNA^{Ala}_2$ species have the same anticodon IGC, the two *iso*-species differ structurally in one hexanucleotide located in the anticodon arm; further structural analysis showed that Gm–Ψ in $tRNA^{Ala}_{2a}$ is replaced by Gm–C in $tRNA^{Ala}_{2b}$ [10]. The minor species, $tRNA^{Ala}_1$, also appears to be conformationally different from other $tRNA^{Ala}$ species as judged by the chromatographic behaviour of the tRNAs on BD-cellulose column.

In the following, we have characterized the changes in $tRNA^{Ala}$ species of the posterior and middle parts of the silk-gland by fractionation of charged and uncharged $tRNA^{Ala}$ species on benzoylated DEAE-cellulose column and by electrophoresis on 9.6% and 20% polyacrylamide gels in urea. The appearance of $tRNA^{Ala}_{2a}$ species in relatively major proportion in the posterior silk-gland during the fibroin secretion phase exclusively suggests that this species is a specific gene transcript associated with the rapid translation of fibroin mRNA.

2. Materials and methods

[^{14}C]Alanine (171 mCi/mmol) and [^{3}H]alanine (23.8 Ci/mmol) were purchased from Amersham-Searle and BD-cellulose (50–100 mesh) from Schwarz-Mann.

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The conditions for raising the *B. mori* larvae from European and Japanese strains and the methods for preparing tRNA and tRNA ligases at the end of the last larval instar (6th day) from the silk-glands have been described [6–8]. tRNA acceptance activity was determined in Hepes (*N*-2-hydroxyethylpiperazine *N*-2-ethane sulfonic acid) standard assay mixtures in the presence of saturating amount of acylation enzyme to effect complete reaction [7]. The trichloroacetic precipitates collected on Millipore filters were counted with 78% and 20% efficiency for [^{14}C] and [^{3}H], respectively.

^{14}C or [^{3}H]alanyl-tRNA from the posterior and middle silk-glands were prepared by scaling up the standard reaction mixtures. The aminoacylated product was isolated using a combined silicic acid and Sephadex G-25 column chromatography [12]. Fractionation of tRNA, either charged or uncharged, on BD-cellulose columns was done essentially as described by Gillam and Tener [13].

Polyacrylamide gel electrophoresis was carried out according to Fradin et al. [14] and as described previously [10] using a 9.6% acrylamide gel–7 M urea (400 × 150 × 1.5 mm) in Tris–borate buffer (0.089 M) pH 8.3, and 20% acrylamide gel–4 M urea (150 × 150 × 3 mm) in the same buffer at 12 V/cm and 9 V/cm for 45 ± 5 h and 55 ± 5 h respectively. tRNA spots were located after staining with 0.2% methylene blue solution in 2% acetate buffer, pH 5.0, or by autoradiography.

Identification of tRNA^{Ala} bands was performed by acylation with [^{3}H]alanine and stabilization by nitrous acid reaction according to Hervé and Chapeville [15]. After staining, the gel strips were cut into bands of 1 mm thickness, digested in 30% H_2O_2 at 60°C overnight, and counted in a Bray's scintillation mixture [16].

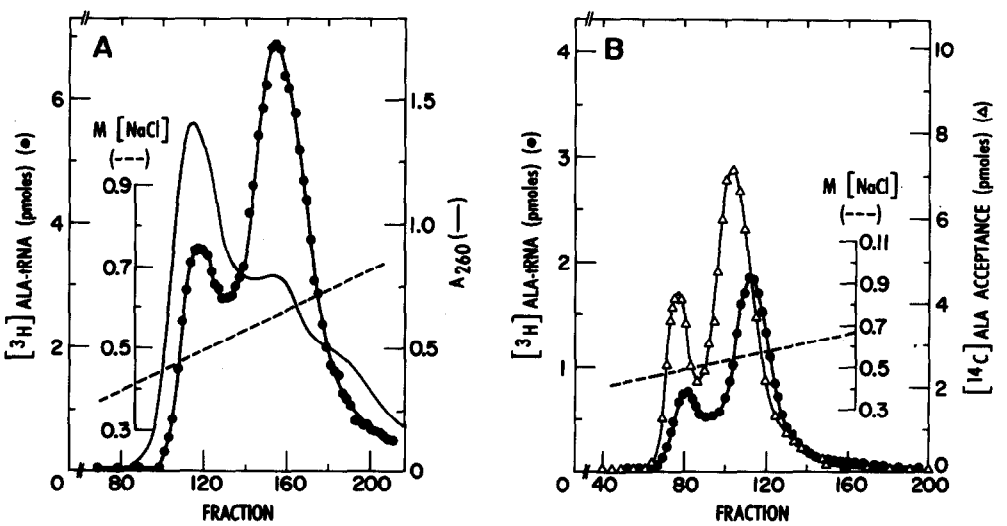


Fig.1. Chromatography on BD-cellulose of [^{3}H]Ala-tRNA and nonacylated tRNA^{Ala} from *B. mori* posterior silk-gland. Posterior silk-gland tRNA was extracted on the 6th day of the last-larval instar.

(A) 374 pmol [^{3}H]Ala-tRNA (2.9 × 10^{6} cpm) dissolved in 0.9 ml equilibrating buffer were loaded on a 0.5 × 40 cm column of BD-cellulose previously equilibrated with 10 ml sodium acetate, pH 4.5, 10 mM $Mg(OAc)_2$, 1 mM β-mercaptoethanol and 0.30 M NaCl. Elution was performed at 4°C with the same buffer using a linear NaCl-gradient as indicated. Fractions of 1.5 ml were collected. Radioactivity was measured by counting 0.1 ml aliquots in Triton–toluene fluor-scintillation mixture. Recovery for radioactivity was 88%.

(B) 17 A_{260}-Units of tRNA and 180 pmol [^{3}H]Ala-tRNA (1.2 × 10^{6} cpm), obtained by charging 0.8 A_{260}-unit of total posterior silk-gland tRNA with [^{3}H]alanine as described in Materials and methods, were applied to a BD-cellulose column (0.5 × 64 cm). Elution was performed as described above. Aliquots of 75 μl were tested for [^{14}C]alanine acceptance using the standard assay detailed in Materials and methods. For radioactivity measurements, aliquots were dissolved in Triton–toluene fluor and counted with 13% efficiency. Recovery was 103% for the [^{3}H]Ala-tRNA.

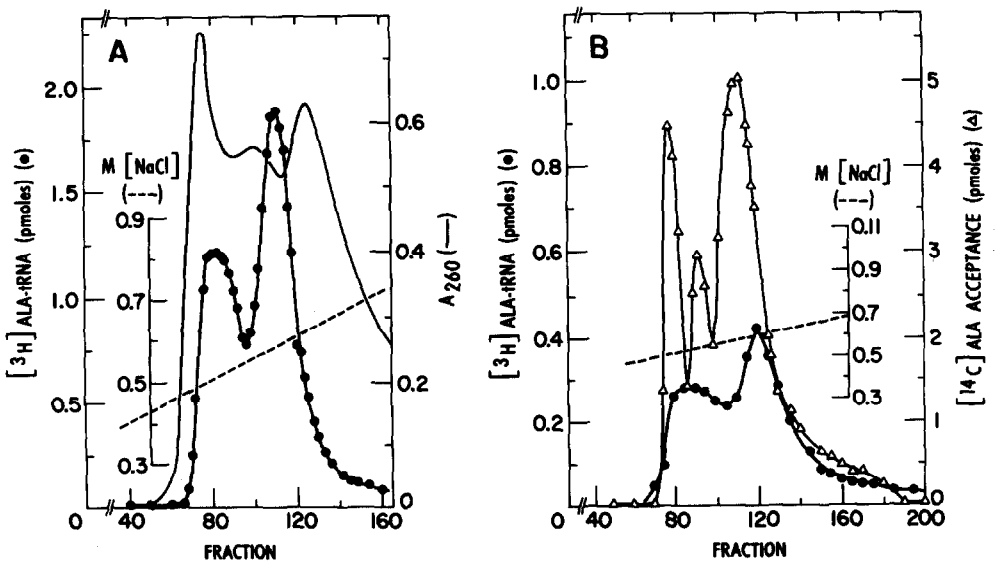
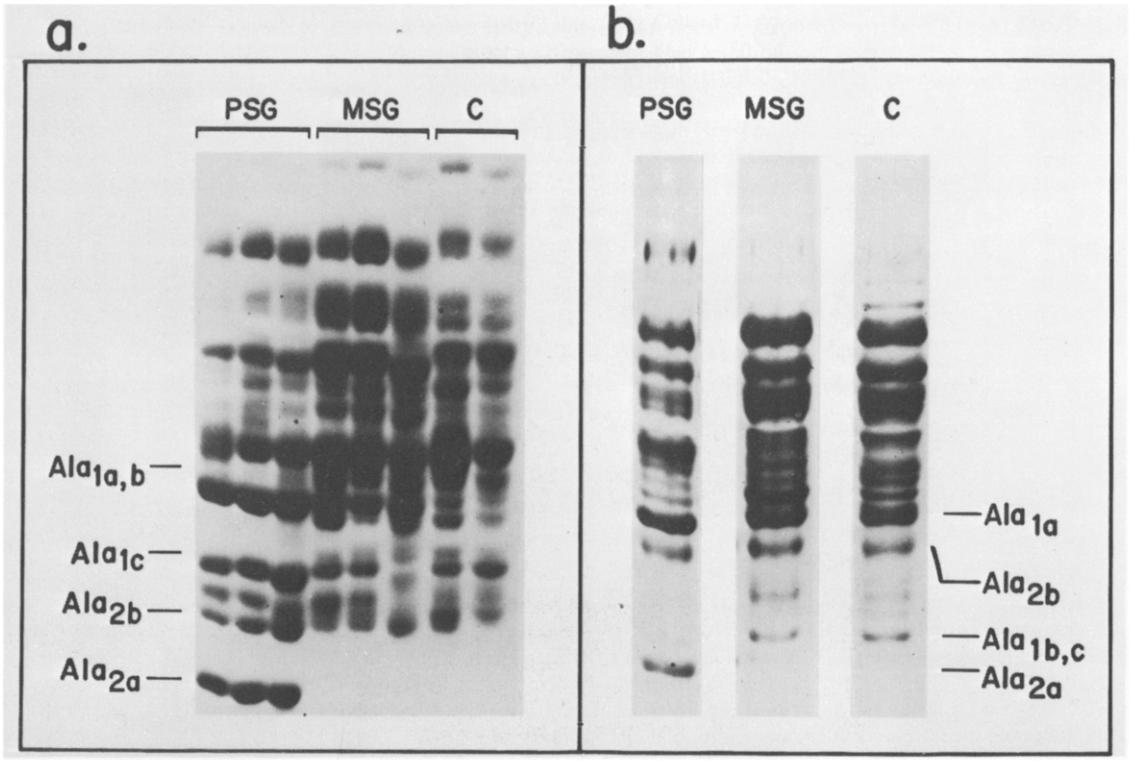
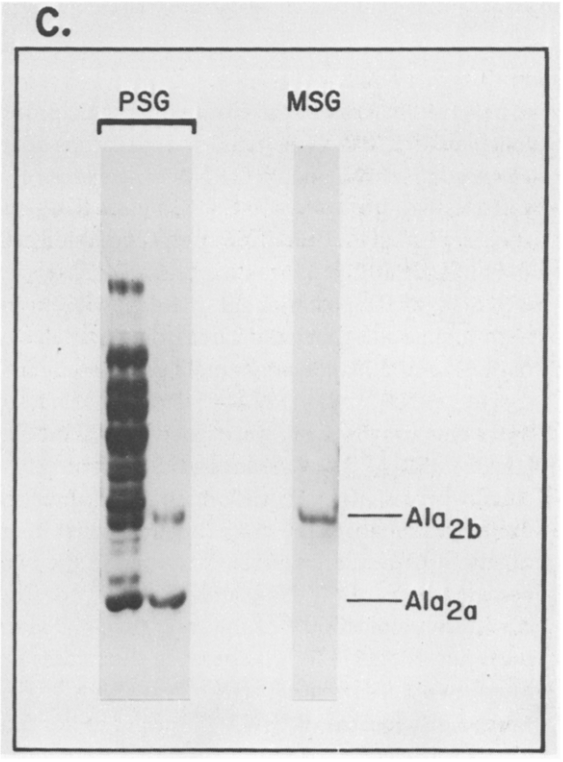


Fig.2. Chromatography on BD-cellulose of [^{14}C]Alanyl-tRNA^{Ala} and non-acylated tRNA^{Ala} from *B. mori* middle silk-gland. Middle silk-gland tRNA was extracted at the 6th day of the fifth-instar.
(A) A sample of 300 pmol [^{14}C]Ala-tRNA (92 000 cpm) was dissolved in 6.5 ml equilibrating buffer and applied to a BD-cellulose column (0.5 × 64 cm). Elution and analysis of the fractions was performed as described in the legend to fig.1A, except that 0.5 ml aliquots were used in the radioactivity measurements. Recovery was 87%.
(B) 148 A_{260}-Units of tRNA and 504 pmol [^{14}C]Ala-tRNA (147 000 cpm) obtained by charging 9.7 A_{260} units of total middle silk-gland tRNA, as described in Materials and methods, were loaded on a 0.5 × 64 cm BD-cellulose column. The chromatography was performed as in the legend to fig.1B. Recovery was 84%.

3. Results

Fractionation of alanyl-tRNA^{Ala} from the posterior and middle portions of the silk-gland on BD-cellulose columns under identical conditions (figs 1 and 2) results in the appearance of two asymetric peaks, peak I and peak II, in a ratio of 23 ± 3% to 77 ± 3%, for the posterior silk-gland, and 38 + 2% to 62 + 2%, for the middle silkgland, respectively. An analysis of the major tRNA^{Ala} peaks (peak II) by electrophoresis on polyacrylamide gel in urea shows (fig.3C) that the posterior silk-gland peak contains two isoaccepting tRNA^{Ala} species, tRNA$^{Ala}_{2a}$ and tRNA$^{Ala}_{2b}$, in a ratio of 2 : 1, but the corresponding peak from the middle part contains only one species, tRNA$^{Ala}_{2b}$, which has identical mobility to that of tRNA$^{Ala}_{2b}$ of the posterior part. A minor tRNA$^{Ala}_{1a}$ species constituted the first peak of the posterior silk-gland tRNA, whereas the middle silk-gland peak I appeared to be made up of two *iso*-species, tRNA$^{Ala}_{1a}$ and tRNA$^{Ala}_{1b}$ (data not shown). The heterogeneity of the peak I tRNA of middle silk-gland is, however, evident in the chromatographic profile of unacylated tRNA^{Ala} (see fig.2b). Separations of total tRNA and identification of tRNA^{Ala} species of the three tissues, posterior silk-gland, middle silk-gland and carcass, on 9.6% and 20% polyacrylamide gels is shown in fig.3. Clearly, the major tRNA^{Ala} species, tRNA$^{Ala}_{2a}$, of the posterior silk-gland was absent in both middle silk-gland and carcass-tissue when examined on 9.6% and 20% polyacrylamide gels. The minor species, tRNA$^{Ala}_{1a}$ and tRNA$^{Ala}_{1b}$, which appeared to move close on 9.6% gel, were resolved well on 20% gel (fig.3b). tRNA$^{Ala}_{1b}$ was absent in the posterior silk-gland whereas tRNA$^{Ala}_{1a}$ was present in all three tissues. Table 1 summarizes the dramatic differences in quantitative distribution in tRNA^{Ala} species in the two compartments of the silk-gland. tRNA$^{Ala}_{2a}$, for example, constitutes almost 60% of the total tRNA^{Ala} in the posterior silk-gland, but is absent in the middle portion, where two-thirds of total tRNA^{Ala} is tRNA$^{Ala}_{2b}$. The relative distribution of tRNA^{Ala} species in the carcass-tissues is somewhat different from that of middle





silk-gland (see table 1 and fig.3). An additional minor species, designated as $tRNA^{Ala}_{1c}$, is detectable in carcass tissues on 9.6% polyacrylamide gel with mobility intermediate to $tRNA^{Ala}_{2b}$ and $tRNA^{Ala}_{1a,b}$. The results thus indicate that $tRNA^{Ala}_{2a}$ is present exclusively in the posterior silk-gland and accumulates at a high level during the fibroin secretion phase.

Upon chromatography of acylated and nonacylated tRNA from the posterior silk-gland a significant variation in the elution pattern is apparent (fig.1B). The peaks obtained by alanine acceptance assay of the

Fig.3. Polyacrylamide gel electrophoresis of total tRNA from posterior and middle silk-glands of the 6 day-old larvae. PSG, posterior silk-gland, MSG, middle silk-gland, C, carcass from European hybrids 200 × 300 and Japanese strains.
(a) Electrophoresis was done on 9.6% acrylamide gel in 7 M urea in Tris–borate buffer, pH 8.3, for 45 h.
(b) Electrophoresis was performed on 20% acrylamide gel in 4 M urea in the same buffer for 60 h at 120 V.
(c) Electrophoresis of tRNA in peak II of BD-cellulose on 20% polyacrylamide gel.

Table 1
Distribution of isoaccepting $tRNA^{Ala}$ species in the silk-gland parts and carcass tissues of *Bombyx mori* L

$tRNA^{Ala}$	Anticodon	Codons	tRNA Content (%)		Carcass
			Posterior Silk-gland	Middle silk-gland	
1a	UGC	GCG	4 ± 1	2	2
1b			–	1.5	2.5
1c			–	–	1.0
2a	IGC	U	14 ± 2	0.5	–
		GCC			
2b		A	7 ± 1	4.5 ± 1	5 ± 1
Total			25	8.5	10.5

The distribution of $tRNA^{Ala}$ species in tissues of 6 day-old fifth-instar larvae of *B. mori* were made by estimating the radioactivity of *N*-nitroso [^{3}H]alanyl-tRNA [15], separated on 9.6% and/or 20% polyacrylamide gel electrophoresis. After staining with 0.2% methylene blue in 2% acetate buffer pH 4.7, the gel strip is cut into bands of 1 mm width, digested in 30% H_2O_2 at 60°C overnight and counted with Bray's scintillation mixture [16]. Total $tRNA^{Ala}$ distribution thus measured was in agreement with ratio of adapted $tRNA^{Ala}$ reported earlier [2]. (See also refs [5,9–11].)

column fractions elute earlier than the corresponding acylated form. The effect of acylation is even more significant in middle silk-gland tRNA (fig.2B). The resolution of the two peaks of alanine acceptor activity ($tRNA^{Ala}_{1a}$ and $tRNA^{Ala}_{1b}$) clearly contrasts with the broad and rather poorly defined peak I comprising of the corresponding acylated alanyl-tRNA species (fig.2A) The alanyl-tRNA peak II is also retarded compared with the elution position of peak II obtained by alanine acceptance. As indicated earlier, the electrophoresis of these tRNAs on polyacrylamide gels shows that this fraction contains a pure $tRNA^{Ala}$ species identical to $tRNA^{Ala}_{2b}$ of the posterior silk-gland. It is known, based on codon binding assays [5] and structural analyses [10,11], that both $tRNA^{Ala}_{2a}$ and $tRNA^{Ala}_{2b}$ species have the same anticodon IGC but differ somewhat in their primary structures outside the anticodon loop (substitution of a pseudouridine residue by cytidine). Structural data on peak I tRNA ($tRNA^{Ala}_1$) species is not yet available.

4. Discussion

Thus, a specific isoaccepting $tRNA^{Ala}_{2a}$ species is present in the fibroin synthesizing compartment of the silk-gland, whereas $tRNA^{Ala}_{2b}$ and $tRNA^{Ala}_1$ species appear to be common to both fibroin and sericin secreting tissues, as well as to the carcass-tissues of the silkworm. $tRNA^{Ala}_2$ Species are capable of decoding GCU, GCC and GCA whereas $tRNA^{Ala}_1$ species decodes GCG [5,9]. $tRNA^{Ala}_1$ therefore differs from $tRNA^{Ala}_2$ at least in its possible anticodon (UGC) structure. However, its earlier elution on BD-cellulose, lower partition coefficient [5,11] and slower electrophoretic migration compared to $tRNA^{Ala}_2$ suggests that there are gross structural differences among the two tRNAs. What is, however, more significant is that in addition to chromatographic and electrophoretic variations among the $tRNA^{Ala}$ species of the two silk-gland portions, striking differences exist in quantitive distribution of the major $tRNA^{Ala}$ species, which most likely reflect their func-

tional involvement in syntheses of fibroin, sericin, and other tissue specific proteins (table 1). The structural differences between the two $tRNA^{Ala}$ species, $tRNA^{Ala}_{2a}$ and $tRNA^{Ala}_{2b}$ [10], would suggest that $tRNA^{Ala}_{2a}$, required to decode the main codon GCU of fibroin mRNA [20], does not arise out of post-transcriptional modifications of $tRNA^{Ala}_{2b}$ and therefore would imply a differential expression of specific $tRNA^{Ala}$ genes. It can be speculated that transcription of $tRNA^{Ala}_{2a}$ genes is specifically derepressed during the fifth-larval instar in order to supplement the pre-existing $tRNA^{Ala}_{2b}$ gene product, which would otherwise be a limiting factor in meeting the rapid translational requirements for fibroin synthesis. Whether the $tRNA^{Ala}_{2a}$ has other specific roles, e.g., regulation of fibroin synthesis, is not known. Current studies are directed toward elucidation of the possible coordination of the exclusive synthesis of this tRNA and the onset of differentiated functions in the posterior silk-gland of *B. mori*.

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